



***Aloe vera* stimulate cell proliferation, cell migration, expression of vascular endothelial growth factor-A (VEGF-A), and c-Jun N-terminal kinase-1 (JNK-1) on fibroblast of diabetic rat models**

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ABSTRACT

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The disturbance of cell migration and cell proliferation, diminished production of vascular endothelial growth factor-A (VEGF-A) and c-Jun N-terminal kinase-1 (JNK-1) are important factors in wound healing process. *Aloe vera* contains active compounds which can help in the wound healing process. The study aimed to investigate the effect of ethanol extract of *A. vera* on cell proliferation, cell migration, VEGF-A and JNK-1 expression of skin fibroblast cells of diabetic rats. The primary skin fibroblast cells were isolated from diabetic Wistar rat and incubated with the *A. vera* extract in various concentrations i.e. 500 (AV500), 250 (AV250), and 125 µg/ml (AV125) for 24, 48 and 72 h. The cell proliferation was examined visually by counting the cells number, the cell migration was observed using *in vitro* scratch assay, whereas VEGF-A and JNK-1 expression were examined using RT-PCR. In 24 and 48h incubation, the cell proliferation of AV500 and AV250 groups had higher number of cells than negative control group, but there was no significant difference ($p > 0.05$). However in 72 h incubation, the cell proliferation of AV500 group ($29.33 \pm 1.28 \times 10^4$ cells/mL) was significantly different compared to negative control group ($22.91 \pm 3.21 \times 10^4$ cells/mL) ($p < 0.05$). In 24 h incubation, the cell migration of AV500 ($78.13 \pm 7.18\%$), AV250 ($73.88 \pm 4.75\%$) and AV125 ($68.80 \pm 17.11\%$) groups were significantly higher than that of negative control group ($53.91 \pm 2.74\%$) ($p < 0.05$). In contrast in 48 and 72 h incubation, there were no significant difference in cell migration ($p > 0.05$). The expression of VEGF-A and JNK-1 after incubation with the AV500 for 48 h, were significantly higher than those of negative control group ($p < 0.05$). In conclusion, *A. vera* increases cell proliferation, cell migration, VEGF-A and JNK-1 expression of fibroblast cells of diabetic rat skin.

ABSTRAK

Gangguan migrasi dan proliferasi sel, juga pengurangan produksi VEGF dan JNK-1 yang merupakan faktor penting dalam proses penyembuhan luka. Lidah buaya (*Aloe vera*) mengandung berbagai senyawa aktif yang dapat membantu dalam proses penyembuhan luka. Penelitian ini bertujuan untuk mengkaji efek ekstrak etanol *A. vera* terhadap proliferasi sel, migrasi sel, ekspresi VEGF-A dan JNK-1 dalam kultur fibroblast kulit tikus diabetes. Kultur sel primer fibroblast diisolasi dari kulit tikus Wistar diabetic dan diinkubasikan dengan ekstrak *A. vera* dengan konsentrasi 500 (AV500), 250 (AV250), dan 125 µg/ml (AV125). Proliferasi diperiksa secara visual dengan menghitung jumlah sel, migrasi sel diamati dengan metode uji gores *in vitro*, dan ekspresi VEGF-A dan JNK-1 diperiksa dengan RT-PCR. Pada inkubasi 24 dan 48 jam, proliferasi sel kelompok AV500 dan AV250 lebih tinggi dibandingkan kelompok kontrol negatif, tetapi tidak bermakna secara nyata ($p > 0.05$). Namun pada inkubasi 72 jam, proliferasi sel kelompok AV500 ($29,33 \pm 1,28 \times 10^4$ cells/mL) berbeda nyata dengan

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JNK-1 expression

kelompok kontrol negatif ($22,91 \pm 3,21 \times 10^4$ cells/mL) ($p < 0.05$). Pada inkubasi 24 jam, migrasi sel kelompok AV500 ($78,13 \pm 7,18\%$), AV250 ($73,88 \pm 4,75\%$) and AV125 ($68,80 \pm 17,11\%$) lebih tinggi secara nyata dibandingkan kelompok kontrol negatif ($53,91 \pm 2,74\%$) ($p < 0.05$). Sebaliknya pada inkubasi 48 dan 72 jam, tidak terdapat perbedaan migrasi sel secara nyata ($p > 0,05$). Ekspresi VEGF-A dan JNK-1 setelah inkubasi dengan AV500 selama 48 jam lebih tinggi secara nyata dibandingkan dengan kelompok kontrol negatif ($p < 0,05$). Dapat disimpulkan, lidah buaya dapat meningkatkan ekspresi VEGF-A dan JNK-1 sel fibroblast kulit tikus diabetes.

INTRODUCTION

Patients with diabetes mellitus (DM) can develop neuropathy which can cause diabetic foot ulcer. The neuropathy caused by disruption of vascular system and sensory nerve damage in extremities area leads to loss of sensation and skin integrity damage.^{1,2} This is a port of the entry for microbe to cause a chronic wound, which is difficult to cure. Moreover patients with severe diabetic foot ulcer can be amputated.³

Hyperglycemia condition changes metabolism of tissue connection because of disturbance in synthesis process and increased the speed of collagen degradation, which results to delayed wound healing process.^{4,5} Delayed diabetic wound healing is related to low number of vascular endothelial growth factor (VEGF) expression and other growth factors which are the main factors of angiogenesis, cell proliferation, and cell migration on wound healing process.^{3,6} Cell migration activity decreased in hyperglycemia due to the excessive production of reactive oxygen species (ROS) that causes oxidative stress and DNA damage, also failure in cell function.⁷⁻¹⁰ Hyperglycemia obstructs c-Jun N-terminal kinase (JNK) phosphorylation which is the downstream of VEGF, as a result there are disturbance on migration activity and cell proliferation.^{9,11-13}

Aloe vera is a medicinal plant traditionally used to treat acute or chronic wound.^{14,15} Topical form of *A. vera* can accelerate healing process of diabetic wound and plays a role in all stages of wound healing process.^{4,16}

Aloe vera is nontoxic and can maintain or increase epithelial cell and fibroblast cell viability.^{17,18} *Aloe vera* contains variety of active compounds such as aloe emodin, β -sitosterol, and acemannan which can stimulate VEGF production in ischemia reperfusion damaged brain of Mongolian gerbil and fibroblast cell culture of gingival rats.^{19,20} *Aloe vera* oral administration significantly stimulates production of TGF- β 1 and bFGF on wound radiation and increases wound contraction, and increase fibroblast migration, also endothelial cells.²¹ This study aimed to investigate the effect of *A. vera* ethanol extract on cell proliferation, cell migration, VEGF-A and JNK-1 expression of fibroblast cell culture of diabetic rat skin.

MATERIALS AND METHODS

Preparation of *A. vera* ethanolic extract

Aloe vera barbadensis Miller was collected from Unit Pelaksana Teknis Daerah (UPTD) Agribisnis Pontianak, West Borneo, Indonesia. The extract was prepared according to Moniruzzaman *et al.*²² after modification. The leaves of *A. vera* were washed in several times then chopped and homogenized by using blender. About one kg of *A. vera* was macerated using two L of 80% ethanol for 24 h. The macerates were then evaporated under reduced pressure using a rotary evaporator. The semi-dried ethanol extract was then freeze-dried at -55°C and transferred to a reagent bottle to be stored in a freezer at -8°C .

Phytochemical screening

The ethanolic extract of *A.vera* was subjected to various qualitative tests for identification of its constituents using thin-layer chromatography (TLC). Separation was performed using silica gel GF₂₅₄ as the stationary phase and mixture of chloroform : methanol : ethyl acetate (5:1:1, v/v/v) as mobile phases. The chromatogram was observed under UV light at wave length 254 and 366 nm and sprayed with citroborate to detect flavonoids in the extract. Mannose examination was performed using high performance liquid chromatography (HPLC). Sample was added one g to volumetric flask and added 10 mL H₂O, furthermore vortexed and centrifuged at 10.000 rpm for 5 min. The product was filtered by Millex 0.45 µm and injected to HPLC as much 50 µL. The chromatographic separation was conducted with MetaCarb 87P column kept in column oven at 85 °C, H₂O as mobile phase at a flow rate of 0.6 mL/min and RID detector for detection.

Animal preparation

Protocol of the study has been approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (ref. KE/FK/422/EC/2016). Male albino rats of the *Wistar* strain weighing ±200 g obtained from the Department of Pharmacology and Therapy, Public Health and Nursing, Universitas Gadjah Mada were maintained on standard rat feed and water *ad libitum*. The animals were fasted for 12 h but had been allowed free access to water. After fasting, DM was induced by intraperitoneal injection of streptozotocin 60 mg/kg body weight (BW) and nicotinamide 120 mg/kg BW in 0.05 M dissolved in citrate buffer (pH 4.5).²³ After three days, blood glucose level were measured and only animal

with glycaemia higher than 250 mg/dL were considered diabetic.

Fibroblast cell isolation and culture

Rats were sacrificed with an overdose of ketamine and xylazine. Fibroblast cells were isolated according to Seluanov *et al.*²⁴ after modification. Underarm skin area of animal were shaved and dissected to collect skin samples. Skin was cut approximately 6 cm² using sterile technique and cultured using explant method. The cells were cultured under standard conditions, with Dubelco's modified Eagle's medium (DMEM low glucose) containing 10% fetal bovine serum (FBS), 1% penstrep, and 0.5% fungizone (GIBCO®, Grand Island, NY, USA), then incubated at 37°C and 5% CO₂. The medium was changed thrice a week and cells were trypsinized at 80% confluence. Passages 4-7 of cell were used for all experiments. The sample then divided into six groups i.e. KN as normal fibroblast cell + low glucose medium (5 mM); K(+) as fibroblast of diabetic rat + high glucose medium (25 mM) + Algisite-M® dressing; K(-) as fibroblast of diabetic rat + high glucose medium (25 mM); AV500 as fibroblast of diabetic rat + high glucose medium (25 mM) + *A. vera* 500 µg/mL; AV250 as fibroblast of diabetic rat + high glucose medium (25 mM) + *A. vera* 250 µg/mL; AV125 as fibroblast of diabetic rat + high glucose medium (25 mM) + *A. vera* 125 µg/mL.

Preparation of solution

About 5 mg of the ethanolic extract of *A. vera* was diluted in 100 µL dimethyl sulfoxide (DMSO; SIGMA®, St Louis, MO, USA) to obtain a stock solution (50.000 µg/mL) and then dissolved in high glucose medium (DMEM 25 mM glucose, FBS 10%, penstrep 1%, fungizone 0.5%) and created in various concentration of *A. vera* (500, 250, 125 µg/mL). Algisite-M® dressing extract solution was created

by using 2 cm² of Algisite-M® dressing (Smith & Nephew®) and incubated in 8 mL of high glucose medium at 37°C for 24 h. Positive control (Algisite-M®) was used for each assay.

Cell proliferation examination

Cell proliferation examination was performed by counting the number of cells in hemocytometer slide Neubauer. Cells were seeded in microplate-96 wells with 10⁴ cells/mL per well in volume 100 µL, and incubated in high glucose medium (DMEM 25 mM glucose) for 24 hours at 37°C and 5% CO₂. Furthermore, cells were incubated with *A. vera* extract concentration 500, 250, 125 µg/mL and Algisite-M® extract in high glucose medium. The number of cells was then counted in 24, 48 and 72 h after incubation.

Cell migration examination

Cell migration test carried out using in vitro scratch assay method. The number of cells to examine cell migration was 5x10⁴ cells per sample in microplate-24 well. Cells were incubated in high glucose medium (DMEM 25 mM glucose) at 37°C and 5% CO₂ for 24 h. Furthermore, cells were incubated with *A. vera* extract concentration 500, 250, 125 µg/mL and Algisite-M® extract in high glucose medium after creating a wound across the cell monolayer with a yellow plastic tip. Cell migration into the wound surface was then measured after 24, 48, 72 h

after incubation and analyzed by ImageJ program.

Expression of VEGF-A and JNK-1 examination

The number of cells to examine VEGF-A and JNK-1 expression was 2x10⁵ cells/mL per well in volume one mL. Cells were incubated in high glucose medium (DMEM 25 mM glucose) at 37°C and 5% CO₂ for 24 h. Furthermore, cells were incubated with *A. vera* extract concentration 500, 250, 125 µg/mL and Algisite-M® dressing in high glucose medium and incubated at 37°C and 5% CO₂ for 48 h. Total RNA isolated by using RNA ISO Plus or GENEzol™ (GZR100) with recommended procedures. RNA were quantified by using spectrophotometer. PCR cDNA mix were carried out by mixed 5x buffer (Toyobo, Cat. No. TRT-101), random primer (TaKaRa, Cat. No. 3801), deoxyribonucleotide triphosphate (dNTP) (TaKaRa, Cat. No. 4030), and Rever TraAce (Toyobo, Cat. No. TRT-101). cDNA product, PCR water, and PCR mixture were mixed and carried out PCR running with conditions: denaturation at 30°C (10 min), annealing at 42°C (60 min), and extension at 99°C (5 min). Gene amplification was performed by using polymerase chain reaction (PCR) method with specific primer. GoTaq® Green Master Mix (Promega, USA) in 12.5 µL were used to amplifications and using total reaction volume total 25 µL. Furthermore, 10 µL amplification products were visualized by using electrophoresis.

TABLE 1. Primer used to examine VEGF-A, JNK-1 dan GAPDH gene expressions

Primer	Urutan
VEGF-A	F: 5'-ACCTCCACCATGCCAAGT-3'
	R: 5'-TTGGTCTGCATTACATCTG-3'
JNK-1	F: 5'-GCCAGTCAGGCGAGAGATTT-3'
	R: 5'-GGACGCATCTATCACCAGCA-3'
GAPDH	F: 5'-ATGACATCAAGAAGGTGGTG-3'
	R: 5'-CATACCAGGAAATGAGCTTG-3'

TABLE 2. PCR conditions used to examine VEGF-A, JNK-1 and GAPDH gene expressions

PCR condition	Pre Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycle	Sample
VEGF-A	94°2 min	94°10 sec	65°30 sec	72°1 min	72°10 min	35x	4µL
JNK-1	94°2 min	94°10 sec	60°30 sec	72°1 min	72°10 min	35x	4µL
GAPDH	94°2 min	94°10 sec	60°30 sec	72°1 min	72°10 min	35x	4µL

Statistical analysis

Statistical analysis was carried out with the SPSS program for windows. Data were expressed as the mean \pm standard deviation (SD). The differences among the mean values from four independent experiments were analyzed with one-way ANOVA followed by post hoc LSD test analysis. A p value <0.05 was considered significant.

RESULTS

Phytochemical screening of *A. vera*

Phytochemical analysis of the *A. vera* extract by TLC showed the presence of compounds on length of wave 366 nm with rate of flow (Rf) value of 0.32; 0.45; 0.64; 0.84; and 0.90, respectively. HPLC examination of *A. vera* extract result showed the level of mannose compound $<32 \mu\text{g/g}$ (FIGURE 1).

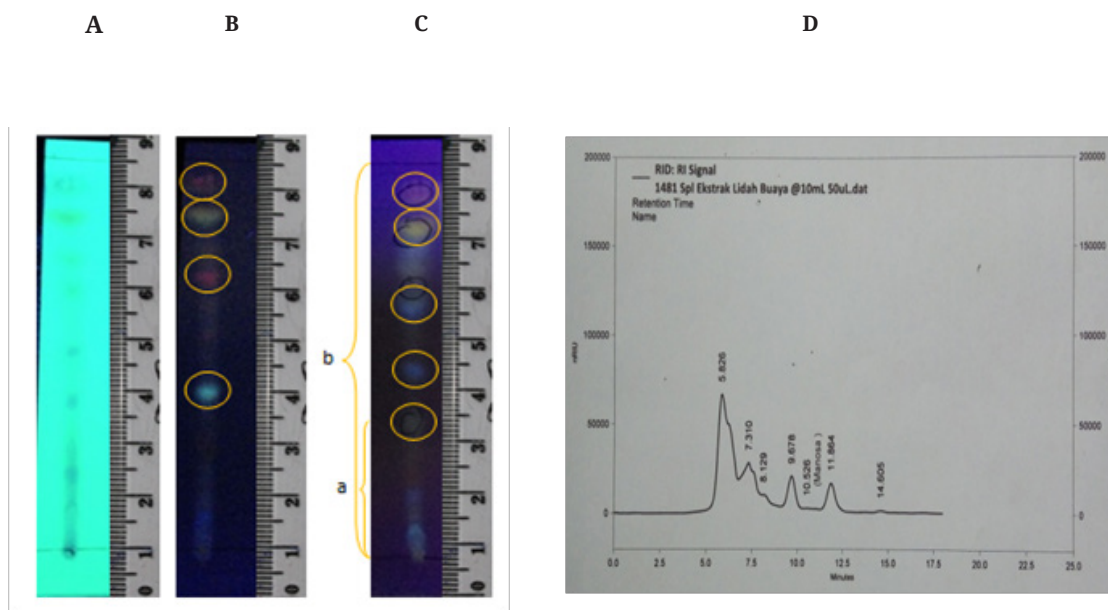


FIGURE 1. The TLC and HPLC chromatogram of *A. vera* extract. (A) Examination by UV 254 nm; (B) Examination by UV 366 nm. (C) Examination by UV 366 nm after Sitroborat spraying. (D) Graphic of HPLC examination (the arrow shows mannose level).

Cell proliferation

Cell proliferation is presented in FIGURE 2. The cell proliferation of the negative control group after 24, 48, 72 h incubations were significantly lower than that normal fibroblast cell group ($p < 0.05$). The cell proliferation of the AV500 and AV250 groups after 24 and

48 h incubations were higher than that the negative control group, but they were not significantly different ($p > 0.05$). However, after 72 h incubation, significantly different between AV500, AV250, and AV125 groups compared to that the negative control group was observed ($p < 0.05$).

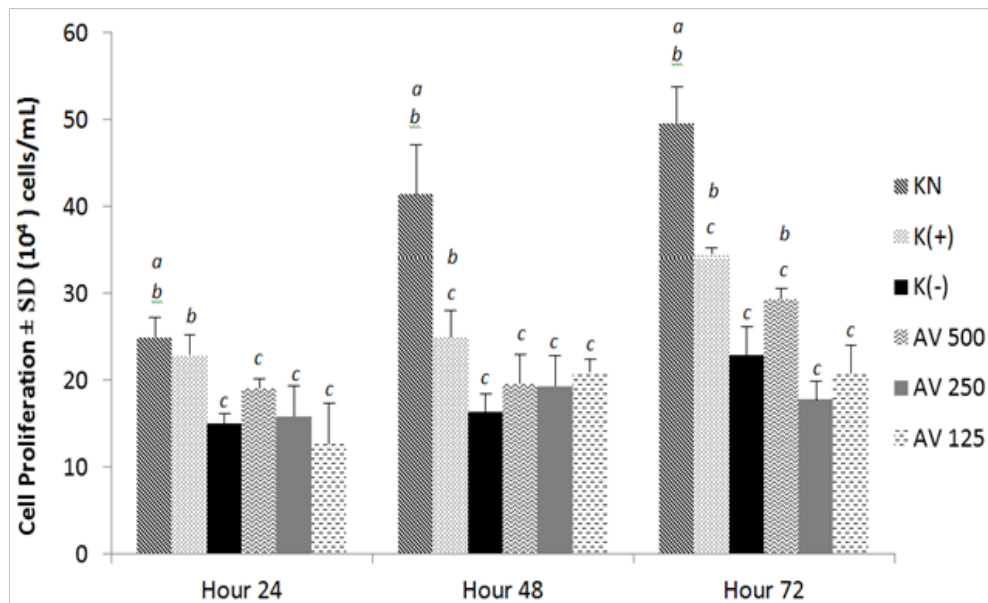


FIGURE 2. Cell proliferation (mean \pm SD 10^4 cells/mL) after 24, 48 and 72 h incubations. One way ANOVA examination in 24h ($p = 0.001$), 48 h ($p = 0.000$) and 72 h ($p = 0.000$). Post hoc LSD: a ($p < 0.05$) compared to positive control; b ($p < 0.05$) compared to negative control; c ($p < 0.05$) compared to normal fibroblast cell control.

Cell migration

The cell migration of the normal fibroblast cell group after 24 h incubation was highest compared to all of the groups ($p < 0.05$). The cell migration of the AV500, AV250 and AV125 group after 24 h incubation was significantly higher

than that the negative control group ($p < 0.05$). However, after 48 and 72 h incubation, no significantly different in all groups were observed ($p > 0.05$). It was indicated that scratch wound has closed at the hour 48 after treatments. The cell migration of all groups are presented in FIGURE 4.

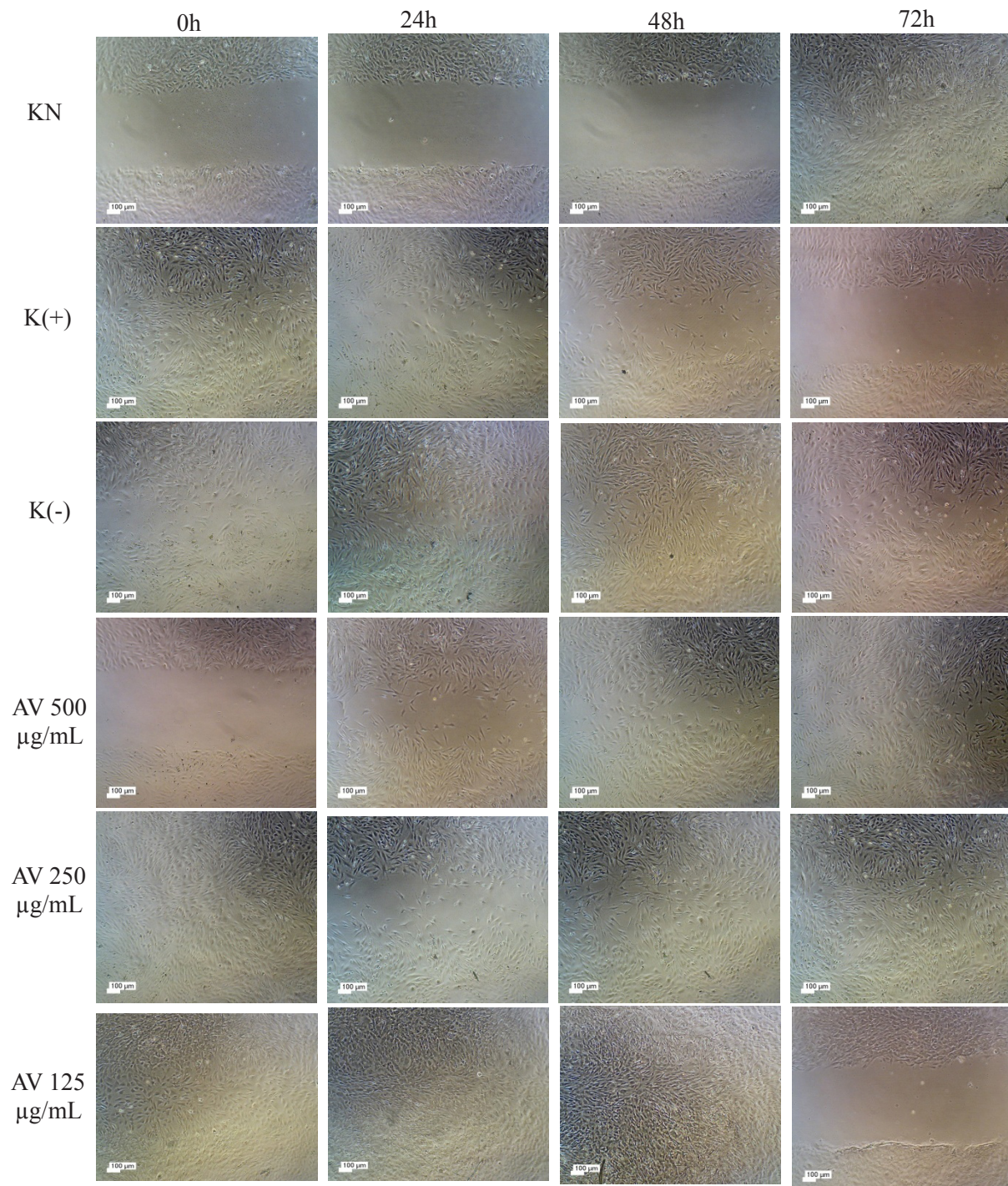


FIGURE 3. Image of cell migration in hour 0, 24, 48, and 72 after treatments. The yellow line was the margin of the scratch. Magnification 40x. The scale of measurement was 100 µm. The migration rate was quantified by the Image-J software and data were expressed as mean ± SD.

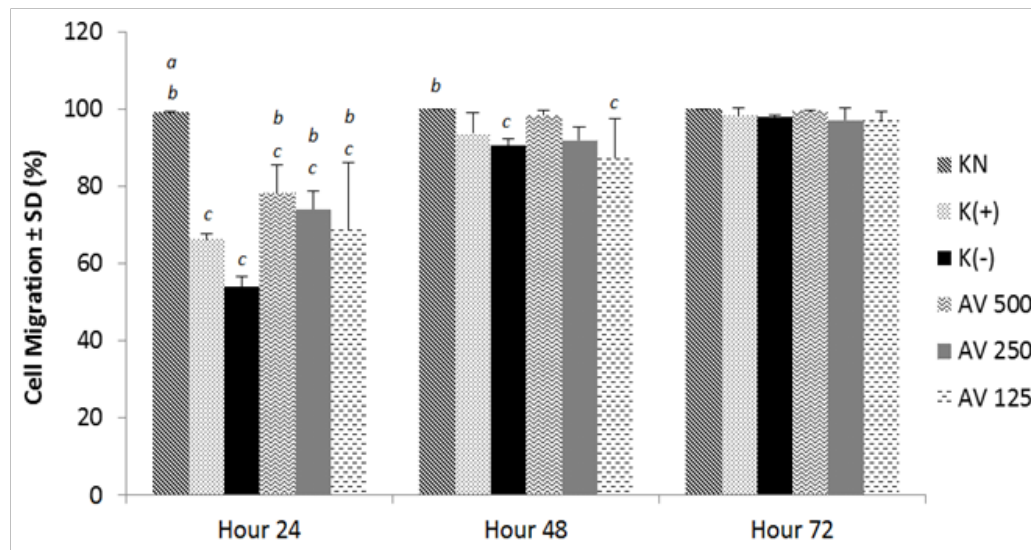


FIGURE 4. Cell migration (mean \pm SD%) in 24, 48, 72 h after incubations. One way ANOVA examination in 24 h ($p = 0.000$), 48 and 72 h ($p > 0.05$). Posthoc LSD: *a* ($p < 0.05$) compared to positive control; *b* ($p < 0.05$) compared to negative control; *c* ($p < 0.05$) compared to normal fibroblast cell control.

VEGF-Aexpression

The normal fibroblast cell control group showed highest VEGF-A/GAPDH expression compared to all of the groups

($p < 0.05$). The AV500 group had higher VEGF-A/GAPDH expression compared to that positive control, negative control, AV250, and AV125 groups ($p < 0.05$).

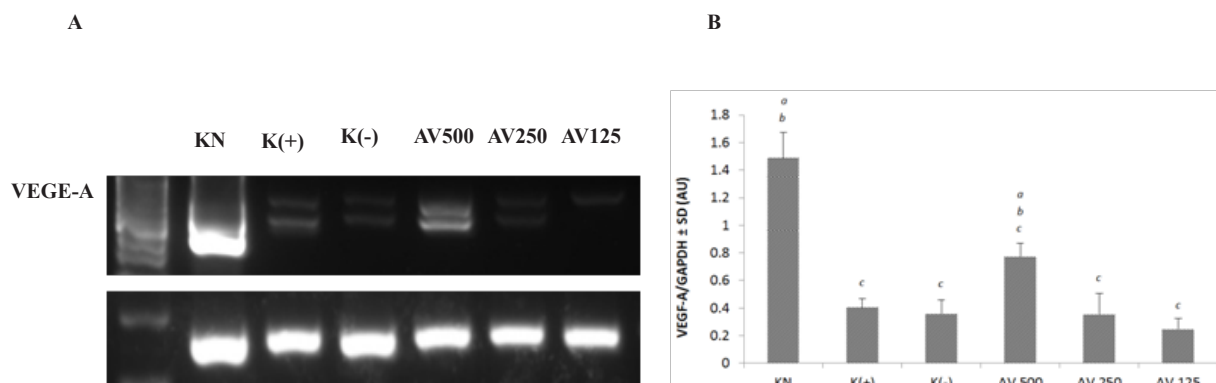


FIGURE 5. (A) Results of electrophoresis VEGF-A and GAPDH gene after 48 hours incubation. (B) Chart bar of mean \pm SD VEGF-A/GAPDH in hour 48. One way ANOVA test ($p = 0.000$). Posthoc LSD: *a* ($p < 0.05$) compared to positive control; *b* ($p < 0.05$) compared to negative control; *c* ($p < 0.05$) compared to normal fibroblast cell control

JNK-1 expression

The normal fibroblast cell control groups showed highest JNK-1/GAPDH expression compared to all of the groups

($p < 0.05$). The AV500 group had higher JNK-1/GAPDH expression compared to that negative control, AV250, and AV125 group ($p < 0.05$).

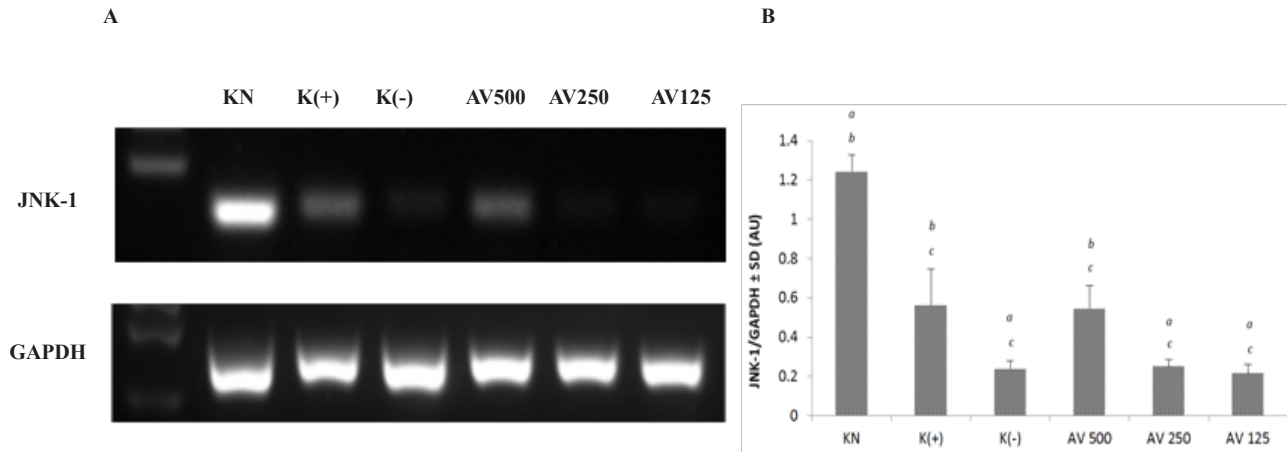


FIGURE 6. (A) Results of electrophoresis JNK-1 and GAPDH gene after 48 hours incubation (B) Chart bar of mean \pm SD JNK-1/GAPDH in hour 48. One way ANOVA test ($p = 0.000$). Posthoc LSD: a ($p < 0.05$) compared to positive control; b ($p < 0.05$) compared to negative control; c ($p < 0.05$) compared to normal fibroblast cell control.

DISCUSSION

In this study, hyperglycemia condition caused cell proliferation and cell migration disturbances, also decreased VEGF-A and JNK-1 gene expression on fibroblast of diabetic rat. It was indicated by significantly fewer cell proliferation of the negative control group compared to that control normal group after 24, 48 and 72 h incubations ($p < 0.05$). In addition, cell migration of negative control group was significantly lower compared to that control normal group ($p = 0.000$) and the scratch wounds of control normal group was almost completely closed compared to the other groups. The VEGF-A and JNK-1 gene expression of negative control group after 24 h incubation was significantly lower compared to that normal control group ($p = 0.000$).

The result of this study was supported by previous studies which

confirmed that hyperglycemia condition caused the cell proliferation and cell migration disturbance.^{7,8,11,12} Moreover, hyperglycemia condition can diminish VEGF production and JNK phosphorylation lead to disturbance of cell proliferation and cell migration on wound healing process.^{8,10,13}

The impairment of cell proliferation on hyperglycemia can be caused by several factors such as increase of L-lactate production,¹¹ inflammatory mediators and advanced glycation end-product (AGE) which stimulates apoptosis through activation of ROS, pro-apoptotic transcription factor FOXO1 and caspase 3.¹² The increase of ROS production affects the protein structure and function, as well as inhibition of cell migration directly by over-activation of the small Rho GTPase Rac1 and altered polarity and morphology of cells.^{7,13} Diminished VEGF production on hyperglycemia occurs by inhibition of JAK-2 and

STAT3 (Signal transducer and activator of transcription-3).²⁵ Hyperglycemia obstructs JNK phosphorylation which is the downstream of VEGF, as a result there are disturbance on cell proliferation and cell migration.^{11-13,26}

Ethanollic *A. vera* extract can stimulate proliferation and migration of fibroblast cell of diabetic rat in this study. The cell proliferation after 24 and 48 h incubations with *A. vera* extract at concentration of 500 and 250 µg/mL were higher than that the negative control group, but they were not significantly different ($p > 0.05$). However, after 72 h incubation, significantly different between *A. vera* extract at concentration of 500, 250 and 125 µg/mL compared to that the negative control group was observed ($p < 0.05$). The cell migration after 24 h incubation with *A. vera* extract at concentration of 500, 250 and 125 µg/mL were significantly higher than that the negative control group ($p < 0.05$). The result of this study was also support by the previous studies which confirmed that *A. vera* can increase cell proliferation and cell migration.^{17,27} The increase of cell proliferation and cell migration occurred in concentration dependent manner. The scratches wounds almost completely closed in each group after 48h incubation. The increase of the cell migration activity is likely occurred before 24 h incubation, so after 24 h the migration has been achieved >50% in each group.

The VEGF-A and JNK-1 expressions after incubation with *A. vera* extract at concentration of 500 µg/mL were significantly higher compared to that the negative control group ($p < 0.05$). It was indicated that *A. vera* can stimulate VEGF production and the other growth factors which has a role in wound healing process as previously reported.^{20,28} The incubation of *A. vera* extract at concentration of 250 and 125 µg/mL on the VEGF-A and JNK-1 expressions were not significantly different compared

to the negative control. This may be related to the small concentrations that do not affect the increased expression of VEGF-A and JNK-1.

The glycoproteins and polysaccharides components in *A. vera* can trigger cell growth and significantly increase cell proliferation and cell migration by stimulating TGF-β1 and bFGF production, also affecting Gap junctional intercellular communication (GJIC) which is the important factors in growth of tissue and organs.^{28,29} Aloesin compound of *A. vera* stimulates cell growth through induce cell cycle protein regulators such as cyclin E, cyclin dependent kinase-2 (CDK2), and cell division cycle-25A (CDC25A) which synergistically up-regulate the cyclin E/CDK2 kinase activity.³⁰ β-Sitosterol compound of *A. vera* increase VEGF expression on brain ischemic reperfusion animals.²⁷

The phytochemical analysis of the *A. vera* extract by TLC showed the presence of compounds on length of wave 366 nm with R_f value of 0.32; 0.45; 0.64; 0.84; and 0.90. It was indicated that *A. vera* extract has various compounds that taken along by mobile phase with various distance based on polarity of compounds. Citroborate spraying was used to examine of flavonoid existence in the *A. vera* extract. The major polysaccharide compound of *A. vera* is acemannan (mannose-6-phosphat) which is composed of one or more polymers of various chain length with molecular weights 30-40 kDa and consists of repeating units of glucose and mannose.³¹ In this research, mannose compound was analyzed by using HPLC and had mannose content <32 µg/gr.

Previous studies reported that *A. vera* accelerates diabetic wound healing^{4,16} by increase the glycosaminoglycans (GAGs), and plasma insulin level, also diminish blood glucose level.³² Acemannan accelerates oral and diabetic wound healing of animal

modelsby stimulates KGF-1, VEGF production, collagen synthesis, and also increase collagen cross link activity for wound contraction.^{20,28} The active acemanan should bind to mannose receptor on the cell surface by recognize polysaccharide chains terminating with mannose, fucose or N-acetylglosamine and stimulate growth factor production and cell proliferationby induce the intracellular signaling pathway.^{20,33} Acemanan promotes skin wound healing and accelerates cell proliferation through stimulate synthesis of protein translationcyclin D1 which is mediated by AKT/mTOR signaling pathway.³⁴

Antioxidant activity of *A. vera* like flavonoid significantly decreases ROS, lipid peroxidase and glycosylated hemoglobin level which are the cause of cell proliferation and cell migration impairment, also diminished of VEGF on diabetic wound.^{7,10,35} This antioxidant activity also has a role in radical scavenging by increase in activity ofsuperoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes.^{7,10,35}

CONCLUSION

Ethanollic extract of *A. vera* increases cell proliferation, cell migration, VEGF-Aand JNK-1expression onfibroblast cell cultured of diabetic rat skin.

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